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TUMOR ANTIGENS RECOGNIZED BY T LYMPHOCYTES

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Abstract

Transplantation experiments have demonstrated that most mouse tumors express antigens that can constitute targets for rejection responses mediated by syngeneic T lymphocytes. For human tumors, autologous cultures mixing tumor cells and blood lymphocytes or tumor-infiltrating lymphocytes have produced $CD8^+$ and $CD4^+$ cytolytic T cell (CTL) clones that recognize tumor cells specifically. Attempts to identify the target antigens by biochemical fractionation of tumor cells up to now have failed, with the important exception of the identification of underglycosylated mucins present on breast and pancreatic carcinomas. Gene transfection approaches have proved more successful. A gene family named MAGE codes for antigens recognized by autologous CTL on a melanoma tumor. These genes are not expressed in normal tissues except for testis. They are expressed in many tumors of several histological types. Differentiation antigens coded by genes such as tyrosinase are also recognized on human melanoma by autologous CTL. The identification of human tumor rejection antigens opens new possibilities for systematic approaches to the specific immune therapy of cancer.

EXISTENCE OF TUMOR REJECTION ANTIGENS

Rodent Tumor Transplantation Antigens

Antigens capable of inducing specific rejection of transplantable tumors by syngeneic hosts were first observed on mouse tumors induced with

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methylcholanthrene (1–3). Mice were inoculated with syngeneic tumor cells, and the resulting tumors were removed by surgery. These mice proved resistant to a second graft of the same tumor. No protection was observed against other syngeneic tumors, indicating that the tumor-specific transplantation antigens carried by individual tumors were different. Later experiments showed that tumors induced by ultraviolet radiation also expressed individually specific antigens causing rejection (4).

Virus-induced tumors bear antigens causing rejection in syngeneic hosts. This was observed with tumors induced by polyoma (5) and SV40 (6). Immunization of mice with virus led to protection against tumor grafts, suggesting that the tumor antigens were viral products. Specific transplantation immunity was also elicited by mouse tumors induced with retroviruses (7).

In striking contrast were the observations made by Hewitt on spontaneous mouse tumors, i.e. tumors observed in the absence of any cancer-inducing treatment. He reported that irradiated tumor cells were unable to generate any immune protection against grafts of the same tumor (8). Later, these results were confirmed with rat tumors (9). The implication was that tumor transplantation antigens were either viral antigens or artefacts observed on rodent tumors induced with massive amounts of carcinogens, conditions unlikely to be fulfilled by the large majority of human tumors.

However, other experiments soon demonstrated that the immune system could also get a hold on such non-immunogenic tumors. This was achieved with the so called tum$^-$ variants that are obtained by treating mouse tumor cell lines in vitro with a mutagen (10). These variants are unable to produce tumors in syngeneic animals because they express very strong new transplantation antigens (tum$^-$ antigens). Tum$^-$ variants were initially derived from a teratocarcinoma cell line that appeared to lack any immunogenicity, even upon injection of living cells followed by surgical removal of the tumor. Nevertheless, mice that had rejected tum$^-$ variants were significantly protected against a challenge with the original teratocarcinoma cells (11). Such a protection conferred by tum$^-$ variants was also observed with the spontaneous tumors of Hewitt, and this protection proved to be specific for the tumor from which the tum$^-$ variants were derived (12). These results indicated that among mouse tumors that are not immunogenic, i. e. incapable of eliciting an immune rejection response, most are nevertheless antigenic, i.e. express an antigen that can be the target of an immune response generated by artificial means. It therefore became plausible that human tumors might also be antigenic, and that their antigens could be rendered immunogenic by appropriate means.

Augmenting Tumor Immunogenicity

The results obtained with tum$^-$ variants were striking because they were obtained with tumors that appeared to be devoid of any immunogenicity. But they were in line with several observations that infection of tumor cells with viruses can increase their ability to elicit immunity against their tumor-specific antigens, a procedure named tumor xenogenization (13–15). A considerable number of subsequent studies reinforced the notion that the immunogenicity of mouse tumors can be increased by modification of the tumor cells. A poorly immunogenic tumor transfected with an influenza virus hemagglutinin gene was shown to generate effective transplantation immunity against the original tumor cells (16). The mechanism by which the addition of strong new antigens ensures an immune response against the weaker antigens present on the original tumor has not been elucidated. But it is quite plausible that the response against the new antigens involves high affinity T lymphocytes that produce cytokines, thereby creating an environment that enables T lymphocytes of lower affinity to respond against the tumor rejection antigens. This hypothesis is supported by a large number of recent reports showing that poorly immunogenic tumor cells transfected with cytokines are readily rejected. This applies to IL-2. CD8$^+$ T lymphocytes appear to be necessary for complete tumor rejection and for subsequent long-term protection against the parental tumor (17–19). Similarly, a weakly immunogenic renal carcinoma transfected with the IL-4 gene was rejected, and mice acquired a T cell dependent immune protection against the original tumor. Injection of these tumor cells transfected with IL-4 could actually cure the mice of a pre-existing tumor (20). Transfer of the IL-7 gene also provokes rejection and protection against the original tumor (21, 22). Tumor cells infected with a recombinant retrovirus carrying the interferon gamma gene showed increased expression of class I MHC molecules and were rejected (23). A nonimmunogenic sarcoma transfected with the same gene induced CD8$^+$ tumor-infiltrating lymphocytes (TIL) that were effective against the parental tumor in adoptive transfer experiments (24). Irradiated B16 melanoma cells that had received retroviral transfer of the GM-CSF gene elicited a long-term protection against the parental tumor, and this protection required both CD8$^+$ and CD4$^+$ T cells (25).

Besides new antigens and cytokines, a third, potentially important, way to improve the immunogenicity of tumor cells has been discovered recently. It involves the transfection of the gene coding for costimulatory molecule B7 (26, 27). All these results suggest new strategies for immunotherapy by active immunization of cancer patients. With the cytokine genes carried by recombinant viruses such as retroviruses, it may even be possible to

infect tumor cells freshly collected from patients at such high efficiency that these infected cells could be reinfused directly as a therapeutic vaccine.

Involvement of T Lymphocytes

By analogy with allograft and viral immunology, the mere occurrence of specific long-term immunity against tumor grafts suggests that T lymphocytes are involved in tumor rejection. Several more precise arguments can be given. First, specific immune memory against tumor challenge can be conferred to syngeneic animals by adoptive transfer of splenic T lymphocytes of immunized animals (28). Further, tumor rejection can be prevented and tumor outgrowth favored by treatments that inactivate T cells; tumor rejection is also often prevented in athymic nude mice (29). Tumors also can be eradicated by adoptive transfer of T lymphocytes. Finally, tumor variants that have escaped rejection in vivo have proven resistant to relevant anti-tumor CTL, indicating that the antigens recognized by CTL play a role in vivo. The two last points are discussed below.

On this basis it seems reasonable to name as tumor rejection antigens not only those antigens defined on the basis of transplantation experiments but also these known to be targets for T lymphocytes that are syngeneic in animals, or autologous in humans.

T LYMPHOCYTES AGAINST MOUSE TUMOR REJECTION ANTIGENS

Anti-Tumor CTL

The first reports describing that CTL recognized mouse tumor cells with unquestionable specificity dealt with retrovirus-induced tumors (30, 31). In general such CTL recognize viral products (32). Interestingly, however, CTL have been described recently that recognize tumors induced by MuLV but not fibroblasts infected with the same virus. Presumably, viral transformation or further progression of the tumor activated some cellular genes that are not ubiquitously expressed (33). CTL have also been obtained against SV40-transformed cells (34) and against adenovirus products (35).

Mouse tumors induced with chemical carcinogens such as mastocytoma P815 also present antigens that are targets for CTL responses (36, 37). These CTL can be obtained by cultivating spleen cells with irradiated tumor cells. They can also be obtained by cultivating tumor infiltrating lymphocytes (TIL) of methylcholanthrene-induced sarcoma tumors in the presence of IL-2 (38). Most of these CTL appear to be specific for the immunizing tumor, suggesting that CTL recognize the individual antigens

of methylcholanthrene-induced tumors. But an anti-P815 CTL recognizes other syngeneic mastocytomas (see below). And a recent report describes CTL obtained against colon carcinoma C26 that are broadly cross-reactive on syngeneic carcinomas and sarcomas (39). CTL were also obtained that are specific for UV-induced fibrosarcoma (40) and against γ radiation-induced tumors (41). Finally, from mice that had rejected tum⁻ variants derived from spontaneous leukemias LEB and LEC, CTL were obtained that recognized the original tumor (12).

Analysis with CTL Clones

Of considerable help for the analysis of tumor rejection has been the availability of long-term clonal cultures of anti-tumor CTL. These CTL clones are usually obtained by limiting dilution and depend on IL-2 and irradiated feeder cells for continuous growth (42).

An important contribution of CTL clones was the possibility to select in vitro antigen-loss variants and to evaluate how many different tumor rejection antigens are recognized by syngeneic CTL on the same tumor. With a panel of CTL clones directed against tumor P815, antigen-loss variants were obtained by mixing a few million P815 cells with a few million cells of various CTL clones (43). Often, survivor P815 cells obtained were permanently resistant to the selecting CTL clone. These cells were still sensitive to most of the other anti-P815 CTL clones. Thus, four P815 antigens could be distinguished (44). A similar analysis has been carried out on a UV-induced tumor, where two distinct antigens were defined (40).

This type of analysis also demonstrated the relevance of the CTL approach for the identification of antigens that serve as targets for tumor rejection. Usually, P815 cells injected intraperitoneally in the syngeneic DBA/2 mice multiply very rapidly and kill the animal in 3–4 weeks. Occasionally, the tumor cells are almost completely rejected by an immune response occurring around day 15 after injection. But invariably, tumor cells that have escaped rejection eventually resume their proliferation and kill the animals. These cells proved resistant to some of the anti-tumor CTL clones, demonstrating that the antigens recognized by these CTL were relevant in vivo (44). The correlation between CTL activity and in vivo results is not absolute though: a CTL directed against a chemically induced colon carcinoma lysed several similar syngeneic tumors and was active against all of them in adoptive transfer. However, no cross-protection was observed with this tumor in transplantation experiments (39).

The clonal approach also made it possible to estimate the number of anti-tumor CTL precursors (CTL-p) in various situations. The first study addressing this question dealt with the anti-MuLV CTL response in mice.

In this system, kinetic analysis of specific CTL formation revealed peak CTL activities in lymph nodes and blood at the time of maximum tumor diameter, whereas intratumoral cells showed peak activity at the onset of tumor regression. On day 10 after virus injection, average CTL-p frequencies were 1/9 in tumor-infiltrating $CD8^+$ T cells vs 1/40 in peripheral blood $CD8^+$ T cells, thus directly demonstrating selective accumulation of CTL-p in the tumor mass (42, 45, 46). For tumor P815, limiting dilution analysis indicated a CTL-p frequency of approximately 1/5000 in spleen cells of immunized animals (47).

Adoptive Transfer

Several studies have dealt with the transfer of anti-tumor responder T lymphocytes obtained in vitro, for the purpose of eradicating established mouse tumors. Erythroleukemia induced by Friend virus (FBL-3) has been the object of particularly thorough experimentation. $CD4^+$ T lymphocytes proved effective notwithstanding the fact that the tumor does not express class II MHC molecules even after treatment with interferon γ (48, 49). Adoptive transfer of $CD8^+$ T lymphocytes worked only if the mice also received IL-2 for 6 days (50). Both bulk responder cells and T cell clones were effective. Cyclophosphamide treatment of the mice was always required. An important recent experiment carried out in this system suggests that adoptive therapy directed against antigens that are not strictly tumor-specific may nevertheless be effective and safe. Transgenic mice were obtained that expressed a low amount of the FBL-3 env gene in their lymphoid system. These mice were inoculated with Friend virus erythroleukemia cells, which expressed a high amount of env product, and they received anti-env CTL. The disseminated tumor cells were eliminated without obvious damage to the lymphoid tissues (51).

Mouse cells transformed by transfection with the E1 region of human adenovirus 5 have also been the object of interesting studies. These cells form tumors only in immunodeficient animals, because contrary to the effect of E1 region of adenovirus 12, the E1 region of adenovirus 5 does not depress MHC class I expression (52). A $CD8^+$ CTL clone directed against an E1A antigen was able to eradicate very large tumors. The CTL persisted for more than 3 months in the mice where they could be restimulated with IL-2 to eliminate a new tumor inoculum (35).

TILs obtained from methylcholanthrene-induced sarcomas have also been used for adoptive transfer. Tumor eradication required cyclophosphamide and IL-2 treatment of the mice. The effector lymphocytes were $CD8^+$ (38, 53). Interestingly, $CD8^+$ TILs that were not lytic but respond specifically to tumor cells by secreting interferon gamma proved effective in vivo (54).

T LYMPHOCYTES AGAINST HUMAN TUMOR REJECTION ANTIGENS

Autologous Anti-Tumor T Cells

The evidence that T lymphocytes of cancer patients can be stimulated in vitro to produce anti-tumor CTL has become gradually more convincing, from the early work where rather low cytolytic activities were observed (55–57) to the rigorous analysis that can now be applied with long-term CTL clones. A lot of attention has been given to melanoma because of the relative ease with which metastatic melanoma cells can be adapted to long-term culture. These cultures provide convenient sources of stimulatory and targets cells for CTL derived from lymphocytes of the same patient (autologous CTL). For more than 50% of melanoma patients, mixed lymphocyte-tumor cultures (MLTC) carried out with their blood lymphocytes and their tumor cell line produce cytolytic responder cells that show appropriate tumor-specificity. These CTL lyse the autologous tumor cells but do not lyse NK or LAK targets such as K562, autologous EBV-transformed lymphoblastoid cells or fibroblasts (58–62). Anti-melanoma CTL have also been derived from lymphocytes of draining lymph nodes (62). CTL have been obtained against other tumor types such as sarcomas and head and neck carcinomas (63, 64).

Tumor-infiltrating lymphocytes (TIL) have also been a source of anti-tumor CTL. Mainly $CD8^+$ T cells were found in TIL populations cultured from melanoma metastases. These TIL exerted lytic activity on autologous melanoma cells, and much less on K562 or mitogen-induced T lymphoblasts (65–67). Long-term TIL cultures containing large amounts of $CD8^+$ cells were obtained, and when some of these TIL lines were transferred to the autologous patient in combination with IL-2 and cyclophosphamide, significant tumor reductions were observed even in patients for whom a prior IL-2 treatment had been ineffective (68, 69). Some TIL lines could be restimulated with freshly thawed tumor cells (69). A similar observation was made with an anti-melanoma CTL clone derived from PBL stimulated in autologous MLTC (70). The targets of these CTL are therefore not artefactual antigens acquired by tumor cells during culture.

Regarding human tumors with viral involvement, CTL that are specific for HTLV-infected cells have been obtained from T cell leukemia patients (71, 72). Remarkably, no CTL directed against Epstein-Barr virus components are observed in patients carrying EBV-positive tumors. In these tumors, the expression of all EBNA products are repressed with the exception of EBNA-1 for which no CTL has ever been derived. This is in sharp

contrast with EBV-induced immunoblastic B cell lymphoma that occurs in immunodepressed patients and expresses many other EBV antigens that can be recognized by CTL (73–75).

It is interesting to note that PBL of B cell lymphoma patients, who were immunized with their idiotypic immunoglobulin conjugated with a foreign protein, made a $CD4^+$ proliferative response when cultivated in the presence of the patient's idiotype (76).

Analysis with T Cell Clones: Multiple Antigens

Long-term CTL clones directed against autologous melanoma cells have been derived by limiting dilution from responder cells of autologous MLTC carried out with PBL (58, 61, 77–79). The vast majority of these CTL clones are $CD8^+$, but $CD4^+$ lytic clones have been obtained (77, 80). CTL clones directed against sarcomas, ovarian and pancreatic carcinomas, and other tumors have also been described (63, 81–83). An interesting recent report describes how irradiated tumor cells from acute myeloid leukemia patients were used to stimulate PBL from HLA identical individuals. This produced CTL clones that lysed the leukemia cells and not EBV-B cells or normal lymphocytes of the patients (84).

Most long-term TIL cultures reported in the literature have not been cloned. An exception is the work of Itoh et al, who cloned by limiting dilution fresh TIL of a melanoma metastasis. Clones were obtained that proliferated for a long time and that lysed the autologous tumor cells and not K562. Some of them were $CD4^+$, others were $CD8^+$ (67). Recently, other anti-melanoma $CD8^+$ TIL clones have been obtained (85).

As in mouse tumor systems, human CTL clones were used to analyze the multiplicity of target antigens on the tumor cells by selecting resistant tumor cell variants. An extensive analysis was carried out on the melanoma cells of patient SK29-AV (79, 83, 86). Four different antigens presented by HLA-A2 could be distinguished. Interestingly it appeared that from the protein corresponding to one of these antigens, a peptide was derived that was also presented by a HLA-B molecule. A similar analysis was carried out on other melanomas (78, 80). On melanoma MZ2-MEL six antigens could be distinguished that were recognized by different CTL clones, a figure now known to be an underestimate. With oligoclonal TIL lines, similar evidence for the presence of at least two distinct antigens on a melanoma line was obtained (87). CTL clones directed against an ovarian carcinoma line recognized three distinct antigens, each of which was lost by various subclones of the tumor line (81).

A limiting dilution approach was used to estimate the frequency of anti-melanoma CTL precursors in melanoma patients. Blood lymphocytes were

stimulated with autologous tumor cell lines in limiting dilution conditions (88). The proliferation of LAK cells in the microcultures could be contained by avoiding the use of feeder cells and by using low concentrations of IL-2. To eliminate the remaining LAK activity, the lytic activity was tested on chromium-labelled autologous tumor cells in the presence of cold K562 competitors. This procedure provided anti-tumor CTL-p frequencies ranging in various patients from 1/1000 to less than 1/200,000. An extension of this approach should prove useful for evaluating the effect of antitumoral immunization in cancer patients, but it is important to recognize that enumeration of tumor specific CTL precursors (CTL-p) by limiting dilution analysis suffers from two limitations. First, it must be established that CTL-p are the only limiting cell type under the assay conditions employed so that Poisson statistics can legitimately be applied. Second, the frequency estimates are minimal estimates which do not take into account possible variations in the clonogenic potential of the cell type under investigation. This reservation was found to be particularly relevant for blood lymphocytes of certain cancer patients and TIL populations, where the overall clonogenic potential of T cells was found to be strongly reduced compared to that of blood T cells from healthy donors (89, 90). In these patients, the frequency estimates of tumor specific CTL-p obtained by limiting dilution analysis may therefore not reflect the actual frequencies of tumor-specific CTL present in lymphoid cell populations.

Common Tumor Rejection Antigens

There is now good evidence that tumor antigens recognized by autologous CTL can be shared by several tumors of the same histological type. A first indication was obtained with CTL clones directed against sarcomas. These clones lysed several allogeneic sarcomas (63, 91). Similar findings were made with melanoma. Some TIL lines directed against melanomas of HLA-A2 patients proved capable of lysing a majority of A2 melanoma lines (92). Definite proof that the target antigens were presented by HLA-A2 was provided by transfection of non-A2 melanoma lines with a construct expressing HLA-A2. The resulting transfectants were lysed (93). Other TIL lines were shown to lyse several melanomas that shared other HLA types (94). Interestingly, when lymphocytes of melanoma patients expressing either HLA-A1 or HLA-A2 were stimulated with allogeneic melanoma sharing the HLA allele, CTL were obtained that lysed the autologous tumor (95). This suggests that, in vitro at least, an anti-allogeneic response does not prevent a response against a tumor antigen. The analysis of cross-reactive CTL has been consolidated with CTL clones. Several laboratories have obtained CTL clones that lyse most A2 mela-

nomas (83, 85). Some of these CTL clones also lyse normal melanocytes, thereby providing an explanation for the presence of their target antigen on most melanomas (96).

IDENTIFICATION OF MOUSE TUMOR REJECTION ANTIGENS

Viral Antigens

The antigens presented to CTL by virus-induced tumors can be identified relatively easily by transfecting cells with the various genes of the viral genome (96a). Cells infected with Friend leukemia virus (FBL-3) bear a gag-encoded antigen recognized on class I molecules and a env antigen recognized on class II (32). For SV40, the large T molecule produces an antigen recognized by CTL (34). CTL directed against adenovirus were shown to recognize a product of transforming gene E1A (35). The antigenic peptide was identified as a decapeptide that binds to H-2D^b (97, 98).

The Meth A Story

For many years the main approach to the identification of tumor rejection antigens involved the fractionation of cell extracts or membrane preparations. The fractions were tested for their ability to confer protective immunity or to stimulate T lymphocytes in vitro. In the light of our present understanding of the nature of antigens recognized by T lymphocytes, and considering the very low number of HLA-peptide complexes that need to be present on any given cell to procure recognition, it is not surprising that this approach has by and large been unsuccessful. But one line of work has provided intriguing results that may eventually be reconciled with present views on antigen recognition by T cells.

In 1986, Appella, Law and coworkers reported the isolation of a tumor specific transplantation antigen from tumor MethA (99). This sarcoma, obtained by methylcholanthrene treatment, elicits a specific immune response that does not confer protection against other methylcholanthrene-induced sarcomas. The purified antigen was isolated from a cytosolic preparation, by evaluating the ability of fractions to confer protection against tumor challenge. The antigen consisted of two proteins of 84 and 86 kDa, whose sequences encoded by different genes were largely but not completely identical (100). Analysis of the sequences indicated that these proteins belonged to the hsp90 family of heat shock proteins (101). The 84 and 86 kDa molecules of MethA conferred an immune protection against MethA and not against other methylcholanthrene-induced sarcomas.

Working on sarcoma MethA also, Srivastava reported at the same time

that cytosol and plasma membrane fractions contained a protein of 96 kDa that conferred immune protection (102). Here again the immunity was strictly specific: a 96-kDa glycoprotein was also isolated from CMS5 sarcoma, and the MethA and CMS5 gp96 immunized only against their respective tumor (102). T lymphocytes from mice immunized with gp96 could transfer specific immune protection and even mediate regression of pre-existing tumors (103). No protective effect against the tumors was obtained with gp96 extracted from normal tissues (104). cDNAs coding for gp96 of MethA, of other sarcomas and of normal tissues were obtained (105). They revealed two important points. First, they proved very similar to members of a family of heat shock proteins (106). Second was the disturbing point that all these cDNAs were identical, providing therefore no basis for the tumor specificity of the antigen (104, 105).

To escape this paradox, a very interesting suggestion was made: gp96 would be not an antigen per se but a carrier for an antigenic peptide that could be transferred to a MHC molecule to become the tumor specific antigen (107). In favor of this hypothesis is the evidence that gp96 has ATPase activity and that peptides can be eluted from purified gp96 fractions (107). It is also in line with the chaperone role of heat shock proteins which implies that they bind to stretches of proteins or peptides. Moreover, a recent report describes that the structure of heat shock proteins may closely resemble that of MHC proteins, including in particular the peptide-binding groove (108). Moreover, gp96 also appears to constitute a very effective way to immunize: only 6 μg of MethA gp96 inoculated in the absence of adjuvant protected mice against tumor challenge, an impressive figure considering that the relevant antigenic peptide must be only one of many carried by gp96 molecules (109). We feel that this interpretation is appealing. However, a measure of the difficulty and the relative confusion still reigning in this field is the recent claim by De Leo and Law (110) that highly purified gp96 fractions of MethA are ineffective, whereas a gp110 that largely copurifies with gp96 represents the effective component. Clearly more work needs to be done before we can feel confident about the interpretation of the gp96 findings.

Antigen P815A: The Genetic Approach

Several antigens have been defined on mouse mastocytoma P815 with syngeneic CTL clones. Two of these antigens, P815A and B, were shown to be linked because, upon selection with CTL, their loss was often simultaneous. These antigens are also relevant in vivo (44). The approach to the identification of the gene encoding antigen P815A and B was based on the transfection of a cosmid library prepared with the DNA of P815 cells into a P815.A$^-$.B$^-$ antigen-loss variant. The transfected cells were tested

for their ability to stimulate the proliferation of an anti-A CTL clone. This led to the identification of transfected cells expressing antigen P815A. These transfected cells were also lysed by anti-B CTL, confirming the linkage between the two antigens. From these transfectants, it was possible to retrieve the cosmid bearing the gene coding for the antigen (111). Thus, a gene named P1A was isolated that codes for both antigens P815A and B (112).

When the sequence of gene P1A cloned from tumor cells was compared to the sequence of the equivalent gene cloned from normal cells of the same mouse strain, no difference was found (112). In addition, the gene isolated from normal tissue transferred the expression of antigens P815A and P815B as readily as the gene cloned from P815 cells. But gene P1A has little or no expression in normal cells, and the antigenicity results from the specific activation of the gene in the tumor cells. P815 being originally described as a mastocytoma, a number of mast cell lines were tested for the expression of the gene. Mast cell line MC/9 and short-term cultures of mast cells isolated from mouse bone marrow were negative on Northern blots. In contrast, a strong signal was obtained with tumorigenic mast cell line L138.8A derived from BALB/c bone marrow by culture in medium containing interleukin-3. Other positive mast cell tumors were found.

The analysis of the gene carried by a P815A$^-$B$^+$ antigen-loss variant indicated that it differed by a point mutation in exon 1. By analogy with previous results obtained with tum$^-$ antigens, this suggested that the region of the mutation coded for the antigenic peptide of antigen P815A (113). Synthetic peptides corresponding to the normal version of the sequence surrounding this mutation were prepared, and one of them proved capable of causing lysis by anti-P815A CTL. Surprisingly, this peptide also sensitized cells to the anti-P815B CTL. It turns out that these two CTL recognize two different epitopes on the same peptide (114).

IDENTIFICATION OF HUMAN TUMOR REJECTION ANTIGENS

MUC-1

By stimulating draining lymph node cells of pancreatic cancer patients with an allogeneic pancreatic tumor cell line, the group of O Finn obtained CTL that lysed most pancreatic tumor cell lines (115). These were CD8$^+$ CTL with $\alpha\beta$ T cell receptors, and they were inhibited by anti-CD3 antibodies. Disturbingly, however, they functioned without MHC restriction. These CTL also lysed most breast tumor cell lines, but not colon carcinomas (116). Knowing that some antibodies recognize mucins on pancreatic and breast tumors and not on the corresponding normal cells, the

investigators purified mucin from pancreatic tumors and found that it stimulated the CTL (116). Similar CTL have been obtained from tumor draining lymph nodes of breast cancer patients. They recognize breast tumor cells but not normal breast epithelial cell lines, even though these also express mucin (117).

Pancreatic and breast mucins are both encoded by gene MUC-1 (118, 119). These molecules have a glycosylated protein core with a repeated motif of 20 amino acids. In breast and pancreatic tumor cells mucin is underglycosylated, and this seems to unveil epitopes of the repeated peptide motif that can be recognized both by specific antibodies and, remarkably, by CTL. This interpretation is supported by experiments carried out with EBV-B cells transfected with gene MUC-1. These cells stimulated the proliferation of anti-mucin CTL clones, but they were not significantly lysed by the CTL unless their glycosylation was inhibited (120). Cells transfected with a construct containing only two tandem repeats of the mucin motif stimulate CTL very effectively, presumably because they are not glycosylated at all. These cells may prove very useful to immunize patients (121).

The MAGE Gene Family

Antigen MZ2-E is one of several antigens recognized by CTL of patient MZ2 on autologous melanoma cell line MZ2-MEL. A cosmid library was prepared with DNA of MZ2-MEL cells, and it was transfected into an MZ2-MEL.E$^-$ antigen-loss variant together with a plasmid conferring resistance to geneticin. Small pools of transfected cells were selected with geneticin and tested for their ability to stimulate the anti-E CTL to release tumor necrosis factor (TNF) (122). Transfectants expressing antigen MZ2-E were identified. From such a transfectant, a cosmid was retrieved that transferred the expression of the antigen. This led to the identification of gene MAGE-1 that codes for antigen MZ2-E (123).

Gene MAGE-1 is about 5 kb long and comprises two short exons and a long third exon. The open reading frame is entirely located in the third exon. Its sequence is unrelated to any known gene. The sequence of the MAGE-1 gene of the melanoma cells is identical to that of a MAGE-1 gene isolated from normal blood cells of patient MZ2 (123). MAGE-1 belongs to a family of twelve closely related genes, all located on chromosome X. Of all these genes, only MAGE-1 produces antigen MZ2-E.

The expression of MAGE genes in various tumors and normal tissues was analyzed by reverse transcription and polymerase chain reaction (PCR) amplification. PCR primers were chosen that are specific for each MAGE gene and that are located in different exons, so as to distinguish by size the amplification products derived from mRNA from those derived

from DNA that might contaminate some RNA preparations (125). About 40% of melanoma tumor samples are positive for MAGE-1, and about 75% are positive for MAGE-2 or 3. A minority of melanoma samples do not express any of the three genes. Almost invariably, the melanomas that express MAGE-1 also express either MAGE-2 or MAGE-3. Besides melanomas, a significant proportion of glioma cell lines (124), breast tumors (125), non small cell lung tumors, and head or neck carcinomas express MAGE-1, 2, or 3. It is worth noting that the level of expression is quite variable from one tumor cell line to another. For tumor samples, the levels observed are minimum estimates, because the proportion of viable tumor cells in these samples is highly variable. In normal tissues, no expression of MAGE-1, 2, and 3 was observed, except in testis, where expression is quite significant. In the absence of appropriate antibodies, it has not yet been possible to determine which testis cell type expresses the gene.

Antigen MZ2-E is presented by HLA-A1 (123). To identify the antigenic peptide, a series of fragments of the third exon of MAGE-1 were transfected into HLA-A1 cells. A sequence of a few hundred nucleotides transferred the expression of antigen E. A peptide encoded by this sequence sensitized very effectively the MZ2-MEL.E$^-$ antigen-loss variant to the anti-E CTL. The most effective peptide proved to be a nonamer (126). The corresponding sequences of the other MAGE genes do not code for the same peptide. The cell lines that express both MAGE-1 and HLA-A1 stimulate the anti-MZ2-E CTL to produce TNF. Some are well lysed by the CTL but others are not, suggesting that their level of expression of gene MAGE-1 produces an insufficient number of peptide-MHC complexes to procure lysis. About 26% of Caucasians express the HLA-A1 allele. Since 40% of melanoma patients bear tumors expressing MAGE-1, about 10% of melanoma patients are expected to express antigen MZ2-E on their tumor.

Some anti-tumor CTL of patient MZ2 recognize a MAGE-1 product on a HLA-C molecule. The relevant peptide is different from the MAGE-1 peptide that combines with HLA-A1. The HLA-C clone 10 allele (127) which codes for the presenting molecule appears to be rather frequent, because the CTL directed against this new MAGE-1 antigen cross-reacted with several allogeneic melanomas (P van der Bruggen, T Boon, in preparation).

Melanoma Differentiation Antigens

HLA-A2 is the most frequently expressed HLA-A allele (49% of caucasians). Tumor rejection antigens presented by HLA-A2 are therefore

more likely to have a wide potential for immunotherapy. As seen above, some A2-restricted CTL are capable of lysing most melanomas of HLA-A2 patients. Such A2-restricted anti-melanomas CTL clones were obtained from patient SK29(AV) (79). Several distinct antigens were defined by immunoselection with these CTL. Two of these antigens, which were named Aa and Ab, appeared to be encoded by linked genes because some antigen-loss variants that were selected with anti-Aa CTL were also resistant to anti-Ab CTL (83).

To isolate the genes coding for antigens Aa and Ab, a genetic approach was followed that differed from the cosmid transfection approach used for MAGE genes. This approach involved the transfection of cDNA libraries into COS cells where the vectors expressing the cDNA multiply episomally to reach a very high copy number (128). A cDNA library was prepared from RNA of melanoma SK29-MEL by cloning the cDNA into vector pcDNAI/Amp. Subgroups of a few hundred clones of the cDNA library were cotransfected into COS cells together with an HLA-A2 gene cloned in the same vector. After a few days the microcultures of transfected COS cells were tested for their ability to stimulate TNF-release by the anti-Aa and anti-Ab CTL. Positive microcultures were obtained and the bacteria of the corresponding pools of cDNA were subcloned. This led to the identification of a cDNA clone coding for antigen Ab (129).

The sequence of the cDNA clone coding for antigen Ab proved almost identical to that of two previously described cDNA that code for tyrosinase (130, 131). This enzyme transforms tyrosine into dihydroxyphenylalanine (DOPA), an intermediate product in the synthesis of melanin. Not surprisingly, the tyrosinase gene is expressed in normal melanocytes. It is not expressed in any other normal tissue including adrenal cells and adrenergic nerve cells that also produce DOPA, because these cells use the enzyme tyrosine hydroxylase, whose sequence is unrelated. The gene that codes for antigen Aa has also been identified recently. It is also expressed specifically in melanocytes and melanomas and is unrelated to any known gene (PG Coulie, V Brichard, A Van Pel, T Wölfel, T Boon, in preparation).

The Peptide Elution Approach

There has been another approach to the identification of antigens recognized by CTL on most HLA-A2 melanomas, involving the isolation and sequencing of the peptides carried by class I MHC molecules on the cell surface. This peptide elution approach is based on the observations that acid treatment of class I molecules releases peptides that can render cells sensitive to the relevant CTL (132, 133). This approach could show that the 'natural" peptides presented by class I molecules range from 8 to 10

amino acids with a majority of nonamers (133–135). The peptide elution approach also made it possible to estimate the number of antigenic peptides present on mouse cells lysed by CTL. The estimate was 200–500 for an influenza nucleoprotein antigen and approximately 100 for a peptide derived from tum$^-$ antigen P198 (134, 136).

Peptides were eluted from HLA-A2 molecules of melanoma cells, and they were fractionated on HPLC. Six fractions were found that rendered cells sensitive to lysis by TIL lines (137). The multiplicity of positive fractions is at least partly due to the presence in these TIL lines of several CTL that recognize different antigens. Similar fractions were eluted from other HLA-A2 melanomas (138, 139). To our knowledge, the peptide elution method has not yet ensured the identification of a peptide recognized by anti-tumor CTL, largely because the sensitivity of the sequencing method limits the approach to the most abundant peptide. But if this difficulty can be resolved, this method could prove very efficient.

Oncogenes as Potential Tumor Rejection Antigens

The analysis of mouse tum$^-$ antigens demonstrated that single point mutations affecting genes expressed by tumor cells could generate very strong rejection antigens (140). The tum$^-$ mutations identified were located in the region coding directly for the antigenic peptides (113, 141, 142). Some of them resulted in an amino-acid change that enabled the peptide to bind to a class I molecule, while others created a new epitope. It is therefore probable that point mutations affecting any gene have a significant probability of generating new antigens recognized by T lymphocytes. An important example are the oncogenes activated by point mutations that occur in many human tumors.

Human lymphocytes stimulated in vitro with peptide 5-16 corresponding to ras mutated in position 12 produced CD4$^+$ T cells that proliferated upon stimulation with the mutated peptide but not with the normal ras peptide (143). T cell clones recognizing such peptides were obtained (144). Mice were immunized with a ras peptide carrying a mutation at position 61 (the mouse and human sequences of this peptide are identical) and their lymphocytes were restimulated in vitro with the peptide. CD4$^+$ T cells proliferated specifically and also recognized cells pulsed with the entire ras protein, showing that the immunizing peptide could be processed naturally (145). Mice were also infected with recombinant vaccinia containing a mutated ras gene, and their spleen cells were restimulated in vitro. CD8$^+$ CTL were obtained that lysed specifically cells infected with recombinant virus containing the mutated ras gene and not those infected with vaccinia containing the normal ras gene. Somewhat surprisingly, a mirror image of

these results was obtained with spleen cells of mice immunized with the normal ras vaccinia construct (146).

Similar results were obtained with p53. Appropriate stimulation with peptides produced human CTL that recognized normal or mutated p53 peptides on T2 or EBV presenting cells (147). Other potential sources of new antigenic peptides are junctional region such as the BCR-ABL region of chronic myelogenous leukemia. Mouse $CD4^+$ T cells could be stimulated in vitro with a BCR-ABL peptide. They recognized cells pulsed with either peptide or the entire p210 BCR-ABL protein (148). It is difficult at this stage to assess the potential of these oncogenes as tumor rejection antigens, but they may be valuable because of their strict tumoral specificity.

SPECIFIC IMMUNOTHERAPY AIMED AT TUMOR REJECTION ANTIGENS

Advantages of Defined Tumor Rejection Antigens

The numerous immunization studies aimed at generating antibody responses against tumor antigens in cancer patients fall outside the scope of this review. For tumor rejection antigens, several active immunization trials involving either allogeneic or autologous irradiated tumor cells have been carried out. Pools of allogeneic melanoma cells with or without adjuvant have been inoculated to melanoma patients (150). Other trials have involved irradiated autologous cells, such as renal carcinoma cells inoculated with lymphokines (151), colon carcinoma cells infected with Newcastle disease virus (152), or haptenized melanoma cells (153). Vaccinia lysates of autologous melanoma cells have also been used (154). In several of these trials, disease-free interval and patient survival showed improvement relative to controls. However, no clear conclusion emerges as how to render these procedures more effective.

The identification of tumor rejection antigens and their encoding genes should open new possibilities for active immunization. The advantages of autologous immunization, namely the certainty that the vaccine bears the antigens of the patient's tumor, and the advantages of allogeneic immunization, namely, that of having readily available vaccines, can now be combined. The patients liable to benefit from immunization with a defined antigen can be identified if a small tumor sample is available. This should be relatively straightforward for patients undergoing surgery. The expression of genes such as MAGE can be ascertained readily by reverse transcriptase-PCR analysis of a very small tumor sample. The patients can also be typed for HLA. Thus, one should know whether a tumor expresses a peptide-HLA combination corresponding to a known tumor antigen

recognized by CTL. For the immunization of these patients, many different forms of the defined antigen can be tried for optimal immunization. Finally, it should become possible to determine whether CTL directed against the relevant antigen have been generated as a result of immunization. Only when this can be achieved regularly should one inquire whether patients with increased numbers of anti-tumor CTL enjoy a more favorable evolution. By proceeding in this way, it should be possible to resolve one by one some of the uncertainties of previous immunization attempts.

Modes of Immunization

To immunize against defined antigens recognized by T cells, there exists a vast array of different modalities. Irradiated cells expressing the relevant antigen represent a first possibility, since it is known that in rodent systems irradiated tumor cells can generate immune rejection responses. However, for human patients, one will have to use available cell lines so that the resulting immunizations will have an allogeneic component. It is difficult to know at this stage whether a strong allogeneic component prevents effective immunization against tumor antigens. If it does it may be possible to build cells that express only the relevant class I MHC molecule, by transfection of cells that do not express class I molecules. To improve the immunogenicity of these cells it may be very useful to inoculate them in medium containing cytokines or to transfect them with cytokine genes such as those of GM-CSF, IL-2, IL-7 or interferon gamma. As discussed above, these cytokines can bring substantial improvements in immunogenicity. Transfecting the gene of costimulatory molecule B7 may also prove very useful.

A second major possibility is that of immunizing patients with purified protein or peptide. Success will probably depend on proper adjuvant formulations, even though immunization of mice with the large T protein of SV40 without adjuvant did generate specific CTL (155). A human papilloma virus (HPV) peptide administered with incomplete Freund's adjuvant protected mice against challenge with HPV-transformed mouse cells (156). But it remains in doubt whether this adjuvant is acceptable for human use. An influenza peptide administered without adjuvant was ineffective, but it became effective after coupling to a lipid moiety (157).

Another use of protein and peptide could be the pulsing of autologous presenting cells such as macrophages or dendritic cells. Cells that express empty class I molecules on their surface may be very effective after pulsing with a peptide (158). Effective autologous antigen presenting cells may also be obtained by infecting macrophages or dendritic cells with defective recombinant retroviruses carrying all or part of the gene coding for the

antigenic peptide (159, 160). This may also be achieved with recombinant vaccinia (161, 162) or adenovirus constructs (163).

Provided recombinant viral constructs are found to meet reasonable safety requirements, they could be used to immunize patients directly. Finally, it may be possible to immunize patients with recombinant BCG or Salmonella bacteria carrying a gene coding for an antigenic peptide (164–166). What a triumph for tumor immunology, if patients could be cured by oral immunization with recombinant bacteria!

Besides active immunization, adoptive transfer of autologous T lymphocyte populations or clones directed against defined tumor rejection antigens can be considered. CTL clones isolated from bone marrow donor lymphocytes and directed against cytomegalovirus antigens have recently proven capable of persisting and functioning in immunodeficient bone marrow recipients (167).

Evaluation of Immunization

It will be very important to evaluate first whether immunization results in increased frequencies of CTL directed against the immunizing antigen, before starting trials where clinical benefit is to be assessed. The evaluation of CTL responses is difficult, even when the relevant antigen is defined. The limiting dilution approach that was used to evaluate anti-tumor CTL-p frequencies depended on the availability of autologous tumor cell lines as stimulators (88). Because such cell lines will not be available for most immunized patients, other modes of stimulation will have to be devised. If the T cell receptor variable gene repertoire directed against a single tumor antigen proves to be narrow, it may also be possible to follow CTL responses by PCR analysis (168).

An alternative approach may be to compare delayed type hypersensitivity (DTH) responses to subcutaneous injections of antigenic peptide before and after immunization. But no correlation between DTH and CTL responses has been observed in at least one clinical trial (169).

Limitations

At this stage, the main task is to demonstrate that effective immunogens can be derived with the available tumor rejection antigens. But one should keep in mind that, even if strong CTL responses are generated, there may be limitations that will affect the clinical efficacy and usefulness of immunization.

A first limitation resides in the lack of complete tumoral specificity of identified tumor rejection antigens. This may be a point of concern for

MAGE genes, and it certainly is for differentiation antigens like tyrosinase. There may be two problems. First, immunization may cause damage to some normal tissues. Careful experimentation with homologous mouse genes may provide useful information in this regard. Second, the affinity of CTL directed against antigens that are not strictly tumor-specific may be low because the high affinity clones may have been eliminated or anergized by tolerance.

A second limitation is that antigen-loss variants or HLA-loss variants may escape rejection. Antigen-loss variants may not present a major problem because most tumors seem to carry several different tumor rejection antigens. Therefore, if immunity can be raised against several of these antigens, the loss of one of them should not result in tumor escape. A more serious problem is the occurrence of HLA-loss variants (170–174). Here two situations should be distinguished. In the first, a few HLA-loss variants are present among antigenic tumor cells. It seems that this situation could be handled by the immune system. Activated CTL not only lyse their target but also produce cytokines like TNF that could kill nearby tumor cells having lost the antigen. Moreover, IL-2 released by activated specific T cells ought to activate NK cells, which have been shown to be particularly effective on targets that do not express HLA (175, 176). There is however another situation for which there will probably be no solution: that of large tumor masses that have lost HLA expression, possibly because of selection brought about by a previous spontaneous immune response.

The number of patients eligible for immunization against defined antigens will likely increase considerably in the near future because of the identification of several new peptide-HLA combinations based on presently known genes and because of the identification of new genes coding for tumor rejection antigens. This progress should also make it possible to immunize any one patient against several tumor rejection antigens, thereby increasing efficacy and limiting tumor escape. We are confident that the methods outlined here will soon lead to the characterization of several other tumor rejection antigens.

It is very difficult to know at this stage whether therapeutic success will be achieved. But there are now clear strategies for the progress of specific anti-tumor immunization, and we anticipate that the next few years will produce a rich harvest of new results that will bring us closer to the moment of truth in specific immunotherapy of cancer.

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